T Helper Type 2-Biased Natural Killer Cell Phenotype in Patients with Pemphigus Vulgaris

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Pemphigus vulgaris (PV) is an autoantibody-mediated bullous disease, but the role of natural killer (NK) cells in its pathogenic process has never been examined in detail. Circulating $CD56^+CD3^-$ NK cells as well as $CD69^+$-activated NK cells were increased in PV patients compared with healthy controls and patients with other autoantibody-mediated autoimmune diseases, including immune thrombocytopenic purpura and myasthenia gravis. Gene expression analysis of highly purified NK cells demonstrated an increased expression of *IL-10* and decreased expression of *IL-12Rβ2*, *perforin*, and *granzyme B ex vivo* in PV patients *versus* healthy controls. The NK cells from PV patients also showed impaired signal transducer and activator of transduction4 phosphorylation upon *in vitro* IL-12 stimulation. Moreover, NK cells from PV patients exhibited reduced IL-10 production in response to *in vitro* stimulation with IL-2/IL-12. Finally, *IL-5* expression in NK cells was exclusively detected *ex vivo* in PV patients with active disease, and was lost in subsequent analyses performed during disease remission. Together these findings suggest that NK cells contribute to a T helper type 2-biased immune response in PV patients through impaired IL-12 signaling and an upregulation of IL-10 and IL-5.

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INTRODUCTION

Pemphigus vulgaris (PV) is an autoimmune bullous disease mediated by anti-desmoglein 3 (Dsg3) autoantibodies that are capable of directly causing acantholysis in the epidermis (Amagai *et al.*, 1991, 1992; Tsunoda *et al.*, 2003; Payne *et al.*, 2005). The production of anti-Dsg3 autoantibodies in PV patients was shown to result from a functional collaboration between Dsg3-specific B and T cells (Nishifuji *et al.*, 2000; Veldman *et al.*, 2004b), but recent accumulating evidence indicates that regulatory mechanisms play an important role in controlling the pathogenic autoimmune response (Kronenberg and Rudensky, 2005). Potential cell types that modulate the autoimmune response include regulatory T cells, natural killer (NK) cells, and natural killer T cells. In this regard, Dsg3-specific T regulatory type 1 cells have the capacity to inhibit Dsg3-specific T helper type 2 (Th2) cells *in vitro* (Veldman *et al.*, 2004a). On the other hand, the roles of NK and natural killer T cells in the pathogenesis of PV have not been evaluated to date. NK cells have long been regarded as an essential component of innate immunity, because they exert nonspecific cytotoxic activity against virus-infected cells and tumor cells (Robertson and Ritz, 1990). However, recent studies have revealed that NK cells are also the source of various cytokines, including both Th1 and Th2 cytokines (Warren *et al.*, 1995; Mehrotra *et al.*, 1998; Hoshino *et al.*, 1999), and they control the adaptive immune response by interacting directly with dendritic cells, T cells, and B cells (Blanca *et al.*, 2001; Mailliard *et al.*, 2003; Zingoni *et al.*, 2004). Natural killer T cells are also capable of producing large amounts of both Th1 and Th2 cytokines upon stimulation and thereby controlling the acquired immune response (Van Kaer, 2005).

In this study, we investigated the involvement of these cell types and their potential regulatory functions in the autoimmune pathogenesis of PV, and found a unique Th2-biased property of NK cells.

RESULTS

Increased and activated circulating NK cells in PV patients

Flow cytometric analysis was performed to examine the proportions of lymphocyte subsets, including those with potential regulatory functions, such as naturally occurring $CD4^+CD25^{high}$ regulatory T cells, $CD56^+CD3^+$ natural killer T cells, and $CD56^+CD3^-$ NK cells from 22 PV patients, including two with active disease, and from 28 healthy controls (Table 1). A statistically significant difference was detected only for the proportion of NK cells ($P=0.003$). A representative flow cytometric finding for NK cells is shown

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ITP, immune thrombocytopenic purpura; MG, myasthenia gravis; NK, natural killer; PV, pemphigus vulgaris; Stat, signal transducer and activator of transduction; Th, T helper

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Table 1. Proportion of lymphocyte subsets in peripheral blood from PV patients and healthy controls determined by flow cytometric analysis[1]

	PV (n=22)	Healthy controls (n=28)	P-value
$CD4^+$	37.6±14.5	37.5±11.9	NS
$CD8^+$	30.4±11.2	27.0±11.0	NS
$CD19^+$	7.5±4.7	9.2±4.0	NS
$CD4^+CD25^{high}$ [2]	2.1±1.1	1.7±0.8	NS
$CD56^+CD3^+$	6.1±7.8	3.4±3.5	NS
$CD56^+CD3^-$	21.3±13.4	10.8±7.0	0.003

PV, pemphigus vulgaris; NS, not significant.
[1]Results are shown as the mean±SD (%). Comparisons were made by the Mann–Whitney *U*-test.
[2]Sample numbers of PV patients and healthy controls were 18 and 27, respectively.

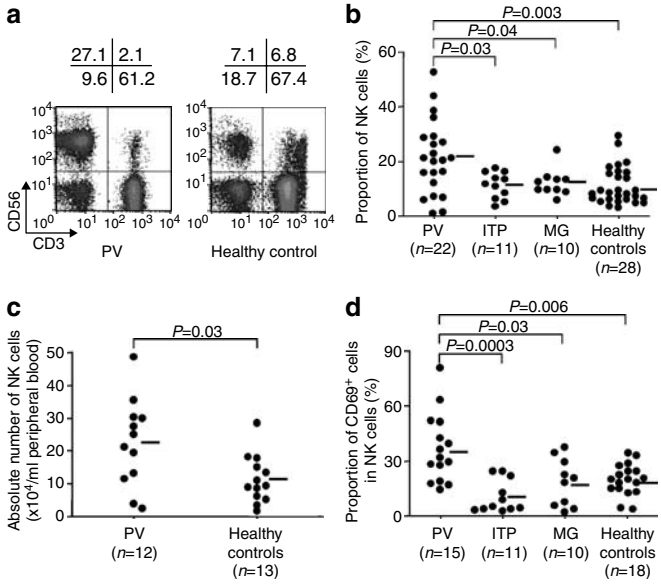

Figure 1. Analysis of NK cells in the peripheral blood from PV patients, ITP patients, MG patients, and healthy controls, by flow cytometry.
(**a**) Representative density plot analysis for evaluating the expression of CD56 and CD3 in a PV patient and healthy control. The cells in the lymphocyte fraction, gated on forward and side scatter, were evaluated. The numbers indicate the proportions of cells in the corresponding quadrants in the lower part. The upper left portion ($CD56^+CD3^-$) corresponds to NK cells. (**b**) The proportion of $CD56^+CD3^-$ NK cells in the circulating lymphocytes of 22 PV patients (two with active disease), 11 ITP patients, 10 MG patients, and 28 healthy controls. (**c**) The absolute number of $CD56^+CD3^-$ NK cells in 1 ml of peripheral blood from 12 PV patients (one with active disease) and 13 healthy controls. (**d**) The proportion of $CD69^+$-activated NK cells in the total NK cells of 15 PV patients (one with active disease), 11 ITP patients, 10 MG patients, and 18 healthy controls. Comparisons were made by the Mann–Whitney *U*-test. Horizontal bars represent the mean values.

in Figure 1a. Additional analyses of samples from 11 immune thrombocytopenic purpura (ITP) patients and 10 myasthenia gravis (MG) patients revealed that the increase in NK cells was unique to PV (Figure 1b). The absolute number of circulating NK cells was also significantly increased in PV patients compared with healthy controls (P=0.03, Figure 1c). We repeatedly assessed the NK cell proportion at different time points (interval 34–161 days) in four PV patients and three healthy controls, and found that the standard deviation of two sequential measurements was <4.0%.

We next evaluated the activation status of NK cells by examining the expression of CD69, a cell-surface marker expressed on activated NK cells (Marzio *et al.*, 1999). The proportion of $CD69^+$-activated NK cells in PV patients was significantly increased compared with the proportion in ITP patients, MG patients, or healthy controls (Figure 1d). These findings together indicate that NK cells were activated and expanded in the peripheral blood of PV patients.

Lack of difference in NK activity between PV patients and healthy controls

We assessed the NK activity in the peripheral blood mononuclear cell fraction from 14 PV patients and 12 healthy controls. NK cells from all the subjects showed cytotoxicity in an effector-to-target ratio-dependent manner. There was no statistically significant difference in the NK activity between PV patients and healthy controls (21.6±14.3% *vs* 17.0±10.5%), even though the proportion and activation status of the NK cells were higher in PV patients compared with healthy controls.

Upregulated expression of the *IL-10* gene and downregulated expression of the *IL-12Rβ2*, *perforin*, and *granzyme B* genes in NK cells from PV patients

To identify genes whose expression levels in NK cells were different between PV patients and healthy controls, we first carried out semi-quantitative PCR to evaluate the mRNA expression levels of 14 genes potentially involved in the regulatory function of NK cells, in NK cell preparations from four PV patients in remission, and four healthy controls (Table S1). A significant difference in the gene expression level between these two groups was detected for *IL-10*, which was upregulated in PV patients and for the *IL-12 receptor β2* (*IL-12Rβ2*), *perforin*, and *granzyme B*, which were downregulated in PV patients, compared with healthy controls.

To confirm these findings, we used the TaqMan® PCR system to quantify the mRNA expression levels in a larger number of subjects: six PV patients with active disease, 13 PV patients in remission, and 11 healthy controls (Figure 2). The expression of *IL-10* was again significantly higher, and the expression of *IL-12Rβ2*, *perforin*, and *granzyme B* was significantly lower in PV patients in remission and with active disease, compared with healthy controls. There was no difference in the expression levels of these four genes between PV patients with active disease and those in remission.

Impaired IL-12 signaling in NK cells from PV patients

The decreased *IL-12Rβ2* gene expression in the NK cells from PV patients could potentially lead to impaired IL-12 signaling. To test this hypothesis, NK cells from five PV patients, including one with active disease, and five healthy controls were stimulated with IL-12 and examined for the

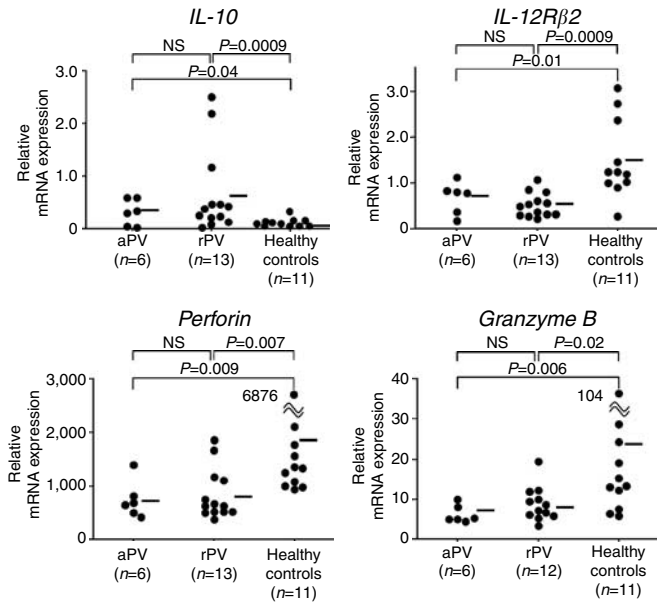

Figure 2. Relative mRNA expression levels of *IL-10, IL-12Rβ2, perforin,* and *granzyme B* in the NK cells from PV patients with active disease (aPV), PV patients in remission (rPV), and healthy controls. Total RNA was prepared from sorted $CD56^{+}CD3^{-}$ cells and subjected to Taqman® quantitative PCR. Individual mRNA expression levels were normalized to the mRNA expression level of *GAPDH*. Comparison was made by the Mann–Whitney *U*-test. Horizontal bars represent the mean values.

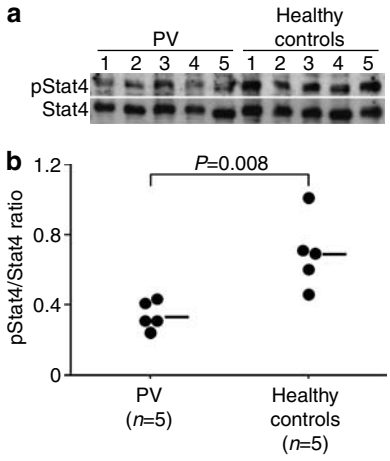

Figure 3. The degree of Stat4 phosphorylation in IL-12-stimulated NK cells in PV patients and healthy controls. (**a**) Sorted NK cells from five PV patients (one with active disease) and five healthy controls were stimulated with IL-12 *in vitro*, and subjected to immunoblot analysis to detect phosphorylated Stat4 (pStat4) and total Stat4. (**b**) The degree of Stat4 phosphorylation in five PV patients and five healthy controls. Comparison was made by the Mann–Whitney *U*-test. Horizontal bars represent the mean values.

phosphorylation of signal transducer and activator of transduction (Stat)4, one of the downstream events selectively induced by IL-12 signaling (Jacobson *et al.*, 1995). Immunoblotting of the cellular lysates of IL-12-stimulated NK cells to detect phosphorylated and total Stat4 revealed that the

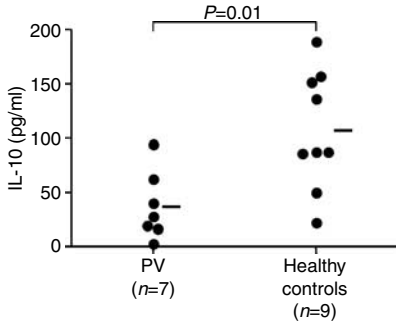

Figure 4. IL-10 production by NK cells from PV patients and healthy controls, upon *in vitro* stimulation with a combination of IL-2 and IL-12. NK cells freshly isolated from seven PV patients and nine healthy controls were cultured with IL-2 and IL-12 for 3 days, and the culture supernatants were subjected to ELISA for measurement of IL-10. Comparison was made by the Mann–Whitney *U*-test. Horizontal bars represent the mean values.

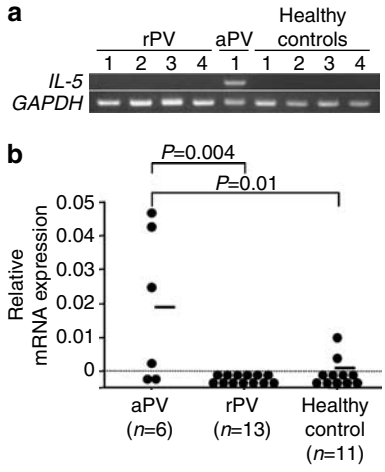

Figure 5. *IL-5* expression in NK cells from PV patients according to the disease activity. (**a**) *IL-5* and *GAPDH* expression by RT-PCR in sorted NK cells from four PV patients in remission, a PV patient with active disease, and four healthy controls. (**b**) *IL-5* mRNA expression levels determined using Taqman® quantitative PCR in six PV patients with active disease (aPV), 13 PV patients in remission (rPV), and 11 healthy controls. Each mRNA expression level was normalized to the *GAPDH* mRNA expression level. A relative mRNA expression level lower than 0 means the value was below the detectable limit. Comparison was made by the Mann–Whitney *U*-test. Horizontal bars represent the mean values.

degree of Stat4 phosphorylation was significantly lower in PV patients than in healthy controls ($P = 0.008$; Figure 3).

Reduced IL-10 production from NK cells upon *in vitro* stimulation with IL-2 and IL-12 in PV patients

To confirm the upregulated IL-10 expression in NK cells from PV patients at a protein level, we measured IL-10 concentration in NK cell lysates using ELISA. However, IL-10 concentration was below the detection limit despite utilization of a highly sensitive commercial kit. NK cells were then stimulated with or without a combination of IL-2 and IL-12, a well-known stimulant for IL-10 production from NK cells

(Mehrotra *et al.*, 1998). IL-10 was undetectable without the stimulation. Interestingly, upon *in vitro* IL-2/IL-12 stimulation, NK cells from PV patients produced significantly less IL-10 than did those from healthy controls ($P = 0.01$; Figure 4).

Upregulated *IL-5* gene expression in NK cells from PV patients with active disease

In patients with multiple sclerosis, *IL-5* expression by NK cells is upregulated in remission, but not in the active disease phase (Takahashi *et al.*, 2001). In contrast, we failed to detect *IL-5* mRNA in the PV patients in remission (Table S1). When the NK cells from additional PV patients were examined, *IL-5* mRNA expression was detected exclusively in the patient with active disease (Figure 5a). The *IL-5* expression by NK cells was further evaluated in 19 PV patients, including six with active disease, and 11 healthy controls using TaqMan® quantitative PCR (Figure 5b). *IL-5* mRNA expression was detected in four PV patients with active disease (67%), but in none of 13 PV patients in remission. Serial analysis in two PV patients with active disease revealed the loss of *IL-5* expression after the disease became quiescent.

DISCUSSION

We demonstrate here that NK cells are quantitatively and phenotypically altered in PV patients as follows: (i) an increased proportion and absolute number of NK cells in circulation; (ii) an increased proportion of $CD69^+$-activated cells; (iii) downregulated *IL-12Rβ2*, *perforin*, and *granzyme B* genes; (iv) impaired IL-12-induced Stat4 phosphorylation; (v) upregulated expression of *IL-10*; (vi) reduced IL-10 production upon *in vitro* IL-2/IL-12 stimulation; and (vii) upregulated expression of *IL-5* in association with active disease status.

The NK cells have been quantitatively analyzed in several autoimmune and inflammatory diseases. The majority of these diseases, including multiple sclerosis, Graves' disease, and psoriasis, are associated with a decreased proportion of NK cells (Kastrukoff *et al.*, 1998; Rojano *et al.*, 1998; Cameron *et al.*, 2003), but PV patients showed an increased proportion. In addition, the *in vivo* activation of NK cells appears to be unique to PV patients, because ITP patients and MG patients lacked this feature. Several lines of evidence indicate that NK cells control the fate of the autoimmune response. Specifically, the depletion of NK cells increases the severity of experimental autoimmune encephalomyelitis, a mouse model for multiple sclerosis, which is a well-documented Th1-mediated autoimmune disease (Zhang *et al.*, 1997). In contrast, NK-cell depletion delays the onset of disease and diminishes the severity of experimental autoimmune MG, a model for autoantibody-mediated autoimmune disease (Shi *et al.*, 2000). These findings indicate that regulatory roles of NK cells in the pathogenic immune response are different among the autoimmune diseases.

NK cells reside not only in the peripheral blood, but also in the spleen and lymph nodes (Ferlazzo *et al.*, 2004), where the interaction between autoreactive T and B cells primarily occurs (Kuwana *et al.*, 2002; Kent *et al.*, 2005). The recruitment of NK cells into the lymph nodes results in induction of the Th1 response by secretion of IFN-γ (Martin-Fontecha *et al.*, 2004). In PV patients, circulating NK cells showed characteristic gene expression profiles, irrespective of the disease status, that is, downregulated *IL-12Rβ2* and upregulated *IL-10*. Because IL-12Rβ2 is a key molecule in Th1 differentiation (Chan *et al.*, 1991; Yoshida *et al.*, 1994; Ma *et al.*, 1996), the downregulated *IL-12Rβ2* mRNA expression and impaired IL-12 signaling in NK cells observed in PV patients may lead to a reduced capacity to induce the Th1 response. Taken together with the upregulated expression of *IL-10* mRNA, it is likely that NK cells contribute to a Th2-biased environment in PV patients. As one of the effects of IL-10 on B cells is to promote production of antibodies, especially of the IgG4 isotype (Jeannin *et al.*, 1998), the upregulated IL-10 expression in NK cells may result in the IgG4-dominant anti-Dsg3 autoantibody response observed in PV patients irrespective of the disease status (Jones *et al.*, 1988; Shirakata *et al.*, 1990; Wilson *et al.*, 1993; Futei *et al.*, 2001).

In contrast to upregulated *IL-10* mRNA expression *ex vivo*, NK cells from PV patients produced less IL-10 than did those from healthy controls upon *in vitro* IL-2/IL-12 stimulation. This phenomenon might be explained by impaired IL-12 signaling observed in NK cells from PV patients. In this case, *in vivo* IL-10 upregulation by NK cells in PV patients is potentially mediated through IL-12-independent pathway. Alternatively, NK cells are already activated and exhausted *in vivo* in PV patients.

We also found an upregulated expression of *IL-5*, another Th2 cytokine, exclusively in PV patients with active disease. Interestingly, this pattern of *IL-5* expression in NK cells in association with the disease status is completely opposite to that seen in multiple sclerosis patients, in whom IL-5 is upregulated in remission, but downregulated in the active phase (Takahashi *et al.*, 2001). Distinct gene expression controls by the NK cells in PV and multiple sclerosis might reflect a difference in the role of Th1/Th2 balance in the disease pathogenesis. As IL-5-producing NK cells inhibit the Th1 response and drive Th2 polarization (Takahashi *et al.*, 2001), the expression of IL-5 by NK cells in patients with active PV further enhances the Th2-biased environment. This might also explain why IgE anti-Dsg3 antibodies are detected in PV patients with active disease, but not in those in remission (Spaeth *et al.*, 2001), because IL-5 is known to enhance IgE secretion (Pene *et al.*, 1988; Vercelli *et al.*, 1989).

In summary, our findings suggest that NK cells may be involved in the pathogenic autoimmune response in PV patients by promoting Th2 polarization, although additional studies using serial samples collected before and after the initiation of the treatment are necessary to confirm our theory.

MATERIALS AND METHODS

Patients and controls

We studied 28 patients (12 men) with PV that were diagnosed on the basis of all the following findings: (1) suprabasal acantholysis by histological examination; (2) IgG deposition on the keratinocyte cell surfaces by direct immunofluorescence; and (3) a positive test for serum IgG anti-Dsg3 antibody. The average age at examination was

51.0±10.8 years. Nine patients (32%) had additional IgG anti-Dsg1 antibody. At the time of blood sample collection, six patients had active disease, and the remaining 22 were in clinical remission, defined as having had no bullae or erosions during the past 2 months, irrespective of treatment.

We also examined 11 patients (two men) with ITP and 10 (two men) with MG to control for autoantibody-mediated autoimmune disease. The average age at examination of the ITP patients and MG patients was 53.7±19.2 and 53.5±18.4 years, respectively. The diagnoses of ITP and MG were based on the published criteria (Drachman, 1994; George *et al.*, 1996). Thirty-five healthy individuals (13 men) were also used as control subjects, and their average age at examination was 47.5±20.1 years. There was no statistically significant difference in sex distribution or age at examination between the PV patient group and the individual control groups. Twenty-two PV patients (76%), four ITP patients (36%), and six MG patients (60%) were taking low-dose prednisolone at the time of blood collection, and the mean daily dosage was 9.3 mg (range 1–19 mg), 8.1 mg (5–10 mg), and 5.8 mg (3–10 mg), respectively. This study was approved by the Keio University Institutional Review Board and conducted according to the Declaration of Helsinki Principles. Written informed consent was obtained from all the subjects.

Flow cytometric analysis

Peripheral blood mononuclear cells were incubated with fluorescence-conjugated mouse monoclonal antibodies, including anti-CD3-FITC, anti-CD4-FITC, anti-CD8-phycoerythrin, anti-CD56-phycoerythrin, anti-CD69-phycoerythrin-cyanin 5.1 (Beckman-Coulter, Hialeah, FL), and anti-CD19-Cy-Chrome (Becton Dickinson, San Diego, CA), followed by analysis with a FACSCalibur® flow cytometer (Becton Dickinson). All analyses were performed on the lymphocyte fraction gated based on the forward and side scatters. The absolute number of the cells of interest was quantified as the number per 1 ml of peripheral blood, based on the ratio to the number of FlowCount® microbeads (Beckman-Coulter). Appropriate fluorescence-labeled isotype-matched control antibodies were included in all the analyses.

NK activity

The nonspecific cytotoxic activity of NK cells was measured by the ^{51}Cr release assay according to a previously described method (Lanier *et al.*, 1983) with some modifications. Briefly, ^{51}Cr-labeled target cells (leukemia cell line K562) were mixed with freshly isolated peripheral blood mononuclear cells at effector-to-target ratios of 1:1, 5:1, 10:1, and 20:1 for 4 hours, and the radioactivity released into the culture supernatants was measured using an automatic gamma counter (Perkin-Elmer Life Sciences, Boston, MA). Each assay was performed in triplicate, and the percent lysis was calculated using the following formula: (experimental release −spontaneous release)/(maximal release in the presence of 1% Triton X−spontaneous release) × 100. NK activity was defined as the percent lysis at an effector-to-target ratio of 20:1.

Isolation of NK cells

NK cells were highly purified by a two-step sorting process using a MACS cell isolation system (Miltenyi Biotech, Bergisch Galdbach, Germany). Namely, $CD3^+$ and $CD14^+$ cells were depleted from peripheral blood mononuclear cells, then $CD56^+$ cells were positively selected. The proportion of $CD56^+CD3^-$ NK cells in the enriched fraction was 98.1±0.3%, as determined by flow cytometric analysis.

Analysis of mRNA expression

Sorted NK cells were used to determine semi-quantitatively their mRNA expression levels of 14 genes (listed in Table S2) that are potentially associated with NK cell function. In brief, total RNA was isolated using an RNeasy mini kit (Qiagen, Hilden, Germany), and treated with AMV RTase XL (TAKARA BIO, Otsu, Japan) in the presence of an oligo-dT primer to generate first-strand cDNA. The cDNA equivalent to 5 ng total RNA was subjected to PCR under the conditions shown in Table S2. The number of PCR cycles was adjusted to assure that the correlation between the amplified product and the amount of input cDNA fell within a linear range. The PCR products were then fractionated on agarose gels and visualized by ethidium bromide staining. The intensity of individual bands that corresponded to the expected molecular size was semi-quantified using NIH Image® available at http://rsb.info.nih.gov/nih-image/. The relative mRNA expression level was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The mRNA expression of the selected genes was further analyzed quantitatively using a Taqman® real-time PCR system with an ABI PRISM® 7900HT (Applied Biosystems, Foster City, CA). cDNA equivalent to 10 ng total RNA was subjected in duplicate to the reactions according to the manufacturer's protocol. Primer pairs and a TaqMan probe for *perforin*, *granzyme B*, *IL-5*, and *GAPDH* were purchased from Applied Biosystems, whereas those for *IL-10* and *IL-12Rβ2* were designed using Primer Express® Software v2.0 (Applied Biosystems) as follows: *IL-10*, sense (5′-GGCGCTGTCATCGATTTCTT-3′), antisense (5′-CTTGGAGCTTATTAAAGGCATTCTTC-3′), probe (5′-CAAGAGCAAGGCCGTGGAGCAGG-3′); and *IL-12Rβ2*, sense (5′-GGGCATTTTCTCAACGCATT-3′), antisense (5′-GCTGGATCTGGAATTTCTCTGCTA-3′), probe (5′-TTCTCCTAGCAGCCCTCAGACCTCAGTG-3′). The gene expression was standardized on the basis of serial dilutions of cDNA prepared from a healthy individual's peripheral blood mononuclear cells that were stimulated with phorbol 12-myrisate 13-acetate (25 ng/ml) and ionomycin (1 μg/ml) (Sigma, St Louis, MO). Relative expression levels were normalized to the expression level of *GAPDH*.

Evaluation of phosphorylated Stat4 in NK cells

The phosphorylation status of Stat4 in NK cells was evaluated as described previously (Toyoda *et al.*, 2004). Briefly, sorted NK cells were incubated with rIL-12 (50 ng/ml; R&D systems, Minneapolis, MN) at 37°C for 30 minutes. The soluble fraction derived from 10^5 cells was fractionated by SDS-7.5% polyacrylamide gel electrophoresis, and subjected to immunoblotting using the Can Get Signal system (TOYOBO, Osaka, Japan) with rabbit anti-phosphorylated Stat4 polyclonal antibodies (Zymed Laboratories, South San Francisco, CA) and peroxidase-conjugated anti-rabbit IgG antibodies (Cappel, Aurora, OH). The antibodies that bound to the membrane were subsequently visualized using Western Lightning™ Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA). After a stripping treatment with Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL), the membrane was re-subjected to immunoblotting using rabbit anti-Stat4 polyclonal antibodies (Santa

Cruz Biotechnology, Santa Cruz, CA). The intensity of individual bands was semi-quantitatively assessed using NIH Image®. The degree of phosphorylation was evaluated as the ratio of the intensity of phosphorylated Stat4 to that of total Stat4.

Quantification of IL-10 production by NK cells

To quantify IL-10 in freshly isolated NK cells, soluble fractions derived from NK cells (10^6 cells) were subjected to highly sensitive ELISA (Quantikine® HS; R&D systems) (sensitivity 0.5 pg/ml). To evaluate IL-10 produced by NK cells upon *in vitro* stimulation, NK cells (10^5/well) were cultured in 96-well flat-bottomed plates with or without a combination of rIL-12 (10 ng/ml) and rIL-2 (100 U/ml; Shionogi, Osaka, Japan) for 3 days. Culture supernatants were harvested and IL-10 concentrations were measured by BD OptEIA Human IL-10 ELISA (BD, Franklin Lakes, NJ) (sensitivity 2.0 pg/ml).

Statistical analysis

All comparisons for statistical differences between two patient groups were performed using the Fisher's two-tailed exact test or Mann–Whitney *U*-test as appropriate.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Table S1. Results of semi-quantitative PCR for 14 immune-associated genes.

Table S2. Primer information.

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